Exhibit 17

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Deposition of genetically engineered human antibodies into the egg yolk of hens

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Abstract

To determine if human immunoglobulins (hIg) are capable of being transported into the hen's egg, 10 μg each of purified hIgG and hIgA were intravenously injected into SC Hyline™ hens and their presence in egg yolk and egg white was determined by ELISA. In both cases deposition into the egg yolk was observed and in the case of hIgA, deposition was also observed in the egg white. Two stably transfected DT40 cell lines secreting recombinant human IgG3 and IgA (rhIgG3 and rhIgA) were injected into laying hens. The DT40 cells colonized the host and rhIgG3 and rhIgA were deposited in egg yolk. Deposition of rhIgA was also observed in the egg white. These data demonstrate that human immunoglobulins and other foreign proteins may be targeted to the chicken's egg. In view of the high rate of reproduction, the short generation interval, the high rates of egg production and the extensive infrastructure to fractionate egg yolk proteins, it should be possible to produce large amounts of foreign protein in the eggs of transgenic chickens.

Keywords: Recombinant human immunoglobulin; DT40 cells; Transgenic chickens; Egg yolk

Abbreviations: Ig, immunoglobulin; hIg, human immunoglobulin; rhIg, recombinant, human immunoglobulin; cIg, (chicken) maternal immunoglobulin; IgYR, IgY receptor; Ab, antibody; rhAb, recombinant human antibody; PBS, phosphate buffered saline; DMEM, Dulbecco's modified eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin.

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1. Introduction

Chicken eggs have long been recognized as a potential source of pharmaceutically important products. In much the same way as transgenic lactating cows, the application of transgenic technology to domestic poultry has been viewed as a

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means of using laying hens as bioreactors. Chicken eggs are ideal receptacles in the context of a bioreactor system, because the yolk and white of eggs are sterile, the technology for fractionating egg yolk and egg white proteins is available and highly automated systems for efficiently producing and collecting thousands of eggs per day are well established. At the forefront of the anticipated uses of transgenic hens as bioreactors, is the use of eggs as efficient repositories of therapeutically useful recombinant human antibodies (rhAb). Approximately 100–200 mg of maternally derived immunoglobulins (Ig) are deposited into the egg yolk with smaller, but still substantial amounts, also deposited into the egg white [1]. Combined with the egg industry's capacity to routinely produce thousands of eggs per day, gram quantities of desirable rhAb might be obtained.

The large-scale production of rhAb and their derivatives is highly desirable due to their tremendous potential for use in therapeutic, research and diagnostic applications [2–8]. During the past two decades, interest in the potential applications of rhAbs has increased because advances in rhAb engineering have enabled the development of antibodies (Ab) with predetermined antigen specificities. In addition, it has been possible to mix and match and mutate domains of the constant regions to create novel molecules with a specific subset of functional properties. For an excellent review on the advances in antibody engineering, the reader is referred to Hayden et al. [9].

While novel and exciting therapeutic applications for rhAb are continually evolving, a cost-effective system for their production remains elusive. Although harnessing the natural Ab production of laying hens for the production of rhAb has been discussed, it has never been demonstrated.

Maternally derived chicken IgG (also referred to as IgY) is deposited in egg yolk via a receptor mediated process [10] whereas maternal IgA and IgM are excluded from yolk. These observations suggest that the receptor that mediates transfer of maternal Ig into the egg yolk is specific for IgY. Since IgY is known to be structurally dissimilar to its mammalian homologue [11], we wished to determine if rhIgG would be recognized by the avian oocyte receptor and if rhIgG would accumulate in egg yolk. Our studies have now shown that upon intravenous administration into laying hens, human IgG, like IgY, is efficiently deposited into the egg yolk and human IgA, unlike its avian homologue, is also deposited, albeit with decreased efficiency. As with chicken IgA, human IgA is deposited into egg white.

To further establish the feasibility of producing rhIg in the eggs of transgenic hens we have produced stably transfected cells from the chicken lymphoma line DT40, with genes encoding rhIgs and intravenously injected the rhAb-producing DT40 cells into so adult laying hens. The transfected DT40 cells synthesized and secreted the rhIg demonstrating that the chicken possesses all of the machinery necessary for efficient rhIg expression and production. Furthermore with this model, we have established that genetically modified populations of avian B cells can exist in vivo in laying hens with the of rhAbs of both γ and α isotypes they produce deposited into the egg yolk and, in the case of α isotypes, into the egg white. The transgenic hen model also provides a simple and efficient means for evaluating the optimal domain structures for transport of rhAb into the egg yolk and egg white. In conjunction with developing transgenic technologies for the suppression of endogenous Ig production [12] and progress towards the production of transgenic hens [13], these studies are the first to support the proposed use of laying hens as bioreactors for the efficient large-scale production of rhAb suitable for use as therapeutic agents.

2. Experimental protocol

2.1. Construction of the transgenes

For the production of functional rhIg of various isotypes, two prototype vectors were developed. One encodes the heavy chain of the immunoglobulin and results in the expression of a murine anti-dansyl variable region joined to the appropriate human heavy chain constant region. The other vector encodes the light chain and

results in the expression of a corresponding murine anti-dansyl variable region joined to a human kappa light chain constant region. Functional anti dansyl mouse/human recombinant antibodies of the desired isotype were produced by co-transfection of these vectors. The rhIgG3 vectors pSV2DHgptV$_{DNS}$HuG3 and pSV184DHneoV$_{DNS}$HuK (heavy and light chain respectively), have been described by Tan et al. [19] and the rhIgA heavy chain vector, pAG5201, has been described by Chuang et al. [18].

2.2. Establishment of chicken pre-B cell lines expressing rhIgs

A chicken B lymphoblastoid cell line, DT40, derived from Hyline SC™ chickens (Hyline, Dallas Center, IA) was used to establish transfected cell lines producing mouse/human chimeric recombinant antibodies (rhIgs). DT40 cells were maintained in culture at $1-10 \times 10^6$ cells/ml in IMDM™ (Gibco BRL) containing 8% (v/v) Bovine Calf Serum (BCS) and 2% (v/v) Chicken Serum (CS). Under optimized electroporation conditions of 200 V, 960 μF and 1000 ms pulse, 1×10^7 DT40 cells were transfected with 20 μg each of linearized heavy and light chain vectors using a BioRad Gene Pulser™ electroporator. Transfected cells were maintained for two days in the above culture media in 96-well micro-titer dishes (2.5×10^4 cells/well) after which selection medium (mycophenolic acid 0.0006% (w/v), xanthine 0.025% (w/v), hypoxanthine 0.0015% (w/v)) was added. Surviving colonies were screened by enzyme-linked immunosorbent assay (ELISA) using dansyl-BSA coated microtiter plates and alkaline phosphatase linked anti-human kappa as the detecting reagents. Strongly positive colonies were then moved into larger dishes for further characterization. Cells from these expanded colonies were labeled by overnight growth in the presence of ^{35}S-methionine. Following overnight growth, culture supernatant and cytoplasmic lysates were prepared and the contents immunoprecipitated using rabbit anti-human Ig and Staph A (IgSorb). Samples were analyzed on 5% polyacrylamide gels without reduction and on 12% gels following reduction. The positions of the bands were determined by autoradiography. Cells from colonies that were observed to produce the desired chimeric antibodies were then maintained in culture medium at $1-10 \times 10^6$ cells/ml. Two cell lines, DT40-hIgG3 and DT40-hIgA, secreting rhIgG3 and rhIgA, respectively, were produced in this manner.

2.3. Intravenous injection of Hyline SC™ hens with hIgG or hIgA

Human derived IgG (hIgG) or IgA (hIgA) (ChromPure Human IgG or IgA, whole molecule. Jackson, PA) was injected intravenously into Hyline SC™ (Hyline, Dallas Center, IA) hens and its deposition into the egg assessed by ELISA. Three hens were injected with 10 μg of hIgG and three were injected with 10 μg of hIgA.

2.4. Intravenous injection of Hyline SC™ hens with DT40-hIgG3 or DT40-hIgA cells

DT40-hIgG3 or DT40-hIgA cells maintained in culture at a concentration of 1×10^6 cells/ml in DMEM™ (Dulbecco's modified eagle's medium) containing 10% FBS (fetal bovine serum) were collected by centrifugation at $300 \times g$ for 5 min and the culture medium removed. Cells were resuspended at a concentration of 10^7 cells/ml in PBS (Dulbecco's phosphate buffered saline, Gibco BRL, Burlington, Ontario) (pH 7.5) and 1 ml of DT40-hIgG3 or DT40-hIgA cells was then injected intravenously into the wing vein of eight Hyline SC™ hens. Hens were weighed prior to injection and then twice weekly to monitor any fluctuations in weight and eggs were collected and assessed for the presence of rhIgG or rhIgA.

2.5. Extraction of human antibodies from chicken egg yolk

All eggs were collected on the day of oviposition and stored at 4°C for further use. The yolk was separated from the albumen and diluted with 40 ml of distilled water (acidified with 0.1 N HCl to a pH of 2.5) and stored at −20°C overnight. Samples were then thawed and centrifuged at $25000 \times g$ at 4°C. The supernatant was then col-

lected and an equal volume of 100%-saturated ammonium sulfate was added dropwise and the samples then stored at 4°C overnight. The samples were again thawed and centrifuged as before. The supernatant was discarded and the pellet resuspended in PBS (pH 7.4).

2.6. Extraction of human antibodies from egg albumen

An equal volume of 100% saturated ammonium sulfate was added dropwise to the thin albumen fraction of individual eggs and samples incubated at 4°C overnight. Samples were then centrifuged as for yolk preparations and the pellet then resuspended in PBS (pH 7.4).

2.7. Detection of human antibodies in yolk and albumen extracts by ELISA

Immunolon™ (Gibco BRL, Burlington, Ontario) 96-well microtiter plates were coated overnight at 4°C with 150 μl/well of a 5 μg/ml solution of goat anti-human IgG (H + L) or goat anti human IgA (Jackson, West Grove, PA) in carbonate buffer (pH 9.6). After overnight incubation the coating mixture was discarded and the plates washed three times with washing buffer (PBS containing 0.05% (v/v) Tween 20). After blocking for 1 h at room temperature (RT) with 300 μl/well of blocking buffer (PBS containing 3% (w/v) bovine serum albumen (BSA) and 0.05% (w/v) Tween 20) the plates were 198 washed three times with washing buffer and 150 μl/well of yolk or albumen extracts were then incubated in the blocked plates overnight at 4°C. Serial dilutions of human immunoglobulin standards (hIgG or hIgA) were also included on the plates to facilitate the computation of hIgG, hIgA, rhIgG3 or rhIgA concentrations in the test samples. Plates were then washed three times with washing buffer and incubated for 2 h at RT with 150 μl/well of horseradish peroxidase-conjugated goat anti-human IgG (H + L) or horseradish peroxidase-conjugated goat anti-human IgA (Jackson, West Grove, PA) at a concentration of 125 ng/ml in diluting buffer (PBS containing 1% (w/v) BSA and 0.05% (v/v) Tween 20). The plates were then washed five times with washing buffer and specific binding revealed by adding tetramethylbenzidine for 15 min at RT and shaking. Color development was stopped by adding 50 μl of 5 M H_2SO_4 and the optical density read by an automated ELISA plate reader (ELISA Plate Reader M550, BioRad Hercules, CA) with a 450 nm filter. The concentrations of anti-dansyl antibodies in yolk and albumen extracts were estimated using dansyl/BSA coated plates.

2.8. Detection of chicken anti-human antibodies produced in hens injected with DT40 hIgG3 or DT40-hIgA cells

Immunolon™ 96-well plates were coated overnight at 4°C with 150 μl/well of a 5 μg/ml solution of dansyl. The plates were then washed three times with washing buffer and blocked for 1 h at RT with 300 μl/well of blocking buffer. The plates were then incubated overnight at 4°C with 150 μl/well of supernatant from DT40-hIgG3 or DT40-hIgA cells grown to confluency (5×10^6 cells/ml) then washed three times with washing buffer and incubated for 2 h with 150 μl/well of individual yolk extracts. Yolk extracts from eggs oviposited on the day of injection were used as negative controls for each bird. The plates were then developed as described above.

2.9. Collection and immunofluorescent staining of blood samples

Blood samples of DT40-hIgG3 and DT40-hIgA injected hens were taken at the time of euthanasia, were diluted 50 times and the cells were collected by centrifugation at $300 \times g$ for 5 min. The cell fraction was then washed twice in staining buffer (PBS (pH 7.5) containing 1% (v/v) FBS and 0.1% (w/v) sodium azide). Samples were then first examined by light microscopy for the presence of cell clusters morphologically similar to that of DT40 cells and then 1×10^6 cells were resuspended in 100 μl of fixation buffer (PBS (pH 7.5) containing 4% (w/v) paraformaldehyde). The cells were fixed at 40°C for 30 min and then washed twice in staining buffer and collected by centrifugation at $300 \times g$ for 5 min. The cells were then

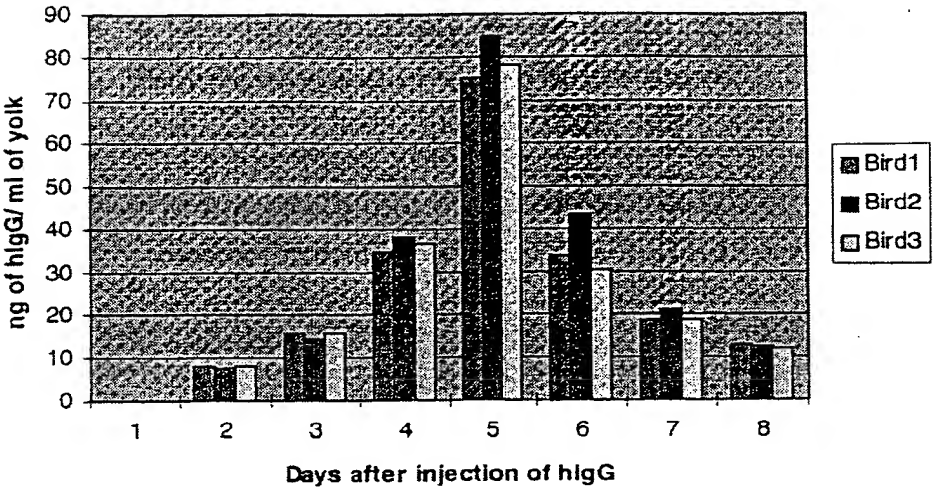

Fig. 1. Mean deposition of hIgG per ml of yolk in eggs laid from hens ($n = 3$) intravenously injected with 10 μg of purified hIgG.

resuspended in 50 μl of a 1:50 dilution of FITC conjugated rabbit anti-human IgG or IgA (H + L) in permeabilizing buffer containing 0.1% (w/v) saponin and incubated at 40°C for 30 min. The cells were then washed twice in permeabilizing buffer, collected by centrifugation as above and resuspended in staining buffer for analysis by immunofluoresence microscopy.

2.10. Statistical analyses

The concentrations of human immunoglobulin in egg yolk after injection of hIgA and hIgG were compared using a repeated measures analysis.

3. Results

3.1. Uptake of intravenously injected hIgG and hIgA into the egg

To determine if human IgG (hIgG) is capable of being transported into the developing chicken follicle, three Hyline SC™ hens were each injected with 10 μg of purified hIgG and its presence in egg yolk and egg white assessed by ELISA. Human IgG was first detected in egg yolk on Day 2, with peak levels of up to 89 ng/ml detected on Day 5 (Fig. 1). No hIgG was detected in the thin albumen extracts indicating that the concentration was less than 3.12 ng/ml (the detection limit of the ELISA assay). Ten μg of human IgA (hIgA) was also intravenously injected into three Hyline SC™ hens to determine if hIgA was also capable of being deposited into the egg. Human IgA was first detected in the egg yolk on Day 2 with peak levels of up to 33 ng/ml detected on Day 5 (Fig. 2A) which was significantly less than the peak deposition recorded for hIgG (Repeated Measures Analysis, $P < 0.01$). Although hIgG was not detected in egg white extracts, hIgA was. Human IgA was first detected in egg white extracts from Day 1 eggs and remained constant at approximately 8 ng/ml of albumen from Days 2 to 8 (Fig. 2B).

3.2. Uptake of rhIgG3 into the egg

DT40 a chicken pre-B cell line derived from Hyline SC™ hens obtained from Dr Craig Thompson. A transfected DT40 cell line, DT40-hIgG3 was produced which secretes a mouse/human chimeric anti-dansyl γ^3 antibody composed of murine anti-dansyl V_H and V_L regions and human $C_L\kappa$ and $C_H\lambda 3$ constant regions (rhIgG3). In order to demonstrate that a hen with populations of endogeneous B cells producing rhIgG3 would transport that rhIgG into the egg, 10^7 DT40-hIgG3 cells were intravenously injected into eight Hyline SC™ hens. Of the eight hens intravenously injected with DT40-IgG3 cells, three de-

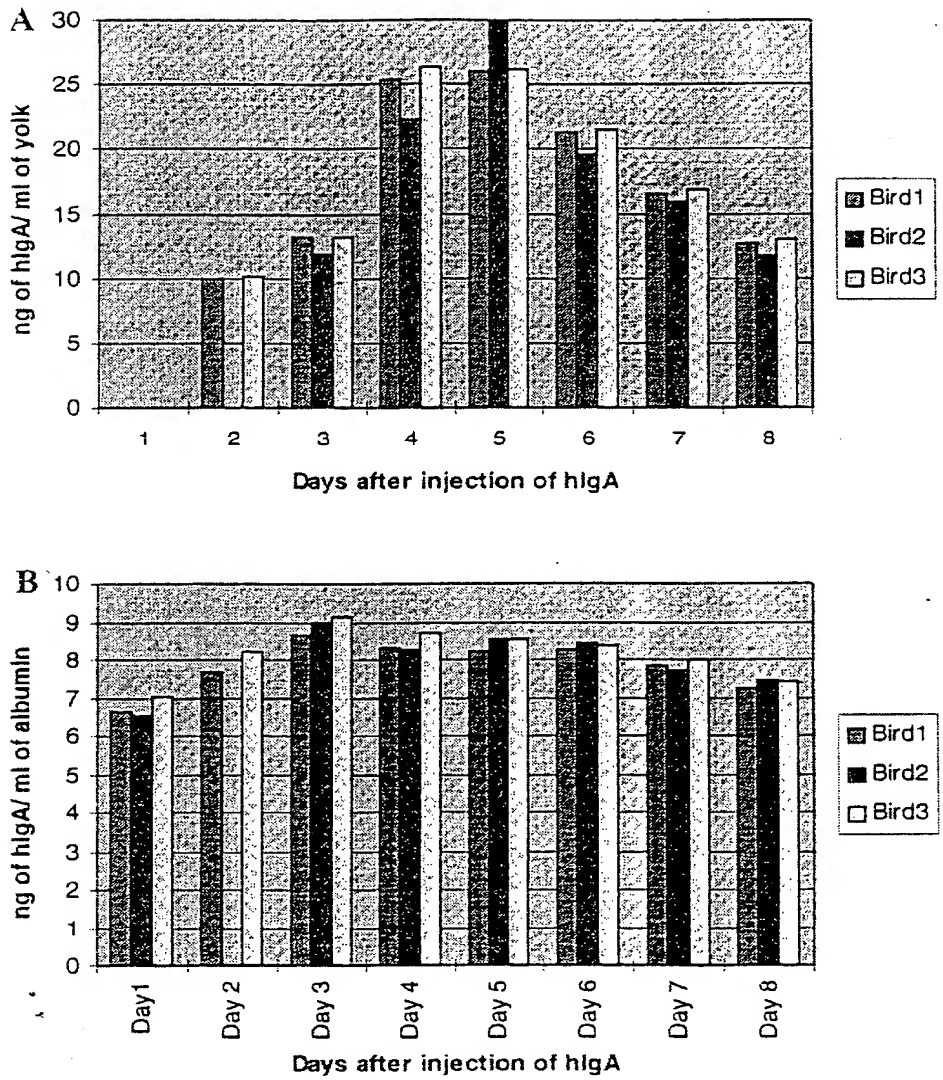

Fig. 2. Mean deposition of hIgA per ml of yolk (A) and albumen (B) in eggs laid from hens ($n = 3$) intravenously injected with 10 μg of purified hIgA.

veloped tumors at the site of injection, indicating that some or all of the cells were injected subcutaneously rather than delivered intravenously. Eggs from hens that developed a tumor at the site of injection had very little rhIgG3 in the egg yolk and none in the thin albumen (data not shown). rhIgG3 was first detected in the egg yolk of the remaining five hens on Day 7 (Fig. 3A and B). The maximum deposition of rhIgG3 in four of these hens was 33 ng/ml of yolk and occurred in eggs laid on Day 10. One of the hens (Bird 6, Fig. 3B) did not lay an egg on Days 10 or 11, and the egg produced on day 12, contained 0.3 μg/ml of rhIgG3 of yolk. Eggs were not laid by this hen on Days 13 and 14 and no rhIgG3 was detectable in eggs laid on Days 15 and 16.

Chicken anti-rhIgG3 was detected in the egg yolk by Day 6 in all hens injected with DT40-IgG3 cells regardless of the presence or absence of a tumor at the site of injection and maximum levels of chicken anti-rhIgG3 in the egg yolk were observed by day 9 (data not shown). All hens

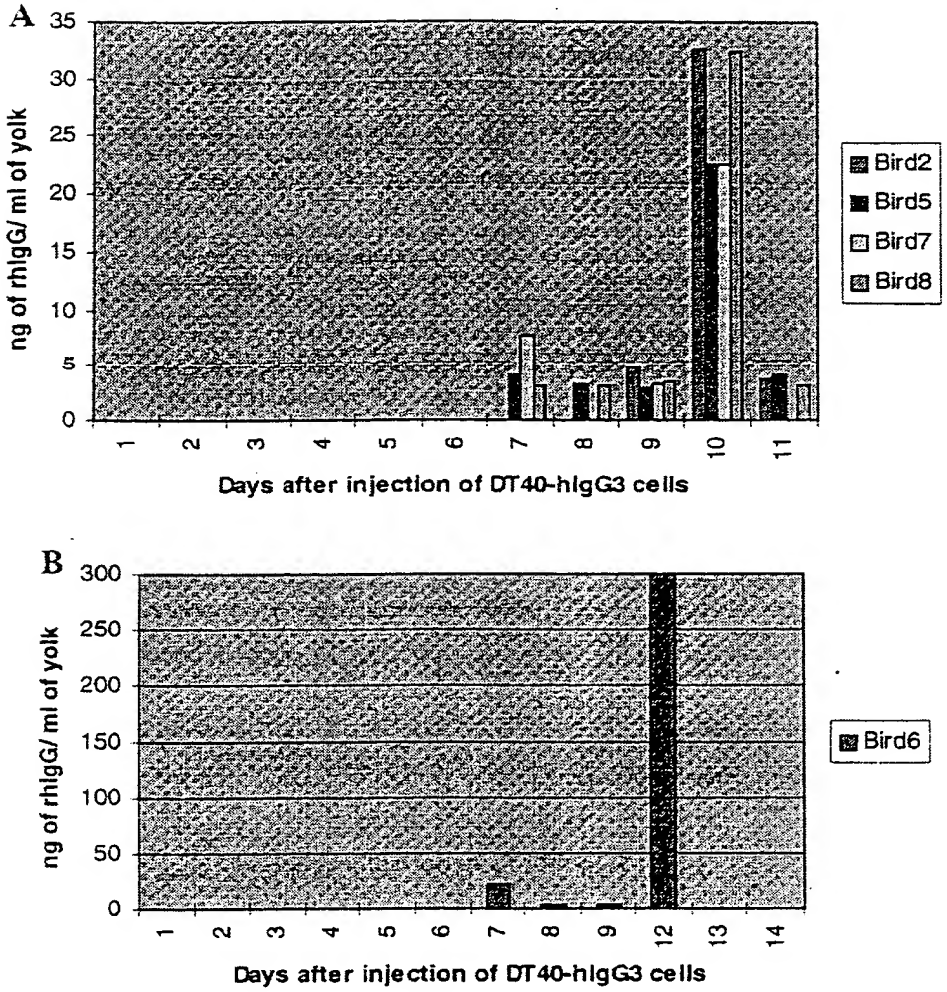

Fig. 3. Mean deposition ($n = 4$) (A) and best deposition ($n = 1$) (B) of rhIgG3 per ml of yolk in eggs laid from hens intravenously injected with DT40-hIgG3 cells, that did not develop tumors at the site of the injection.

intravenously injected with DT40-IgG3 cells, were euthanized on Day 17. On autopsy, no internal tumors were observed in any of the injected birds. To determine if the DT40-IgG3 cells had been maintained as a leukemia, blood samples were taken from the birds and assessed by both light microscopy and by immunofluorescence staining for the continued presence of DT40-IgG3 cells. No DT40-IgG3 cells were observed in blood samples taken from hens that had developed tumors at the site of injection, though cells derived from these tumors, were successfully reestablished in culture and shown to continue production of rhIgG3 (data not shown). In hens that did not develop tumors at the site of injection, clusters of cells that appeared morphologically similar to the DT40-IgG3 cells were observed in diluted blood samples (Fig. 4A). These were confirmed to be DT40-IgG3 cells by immunohistochemical staining for intracellular hIgG (Fig. 4B).

3.3. Uptake of rhIgA into the egg

Similar to DT40-IgG3 cells, a transfected DT40 cell line, DT40-hIgA, was produced that secretes mouse/human chimeric anti-dansyl α antibodies (rhIgA). In order to demonstrate that a hen with populations of transfected B cells producing

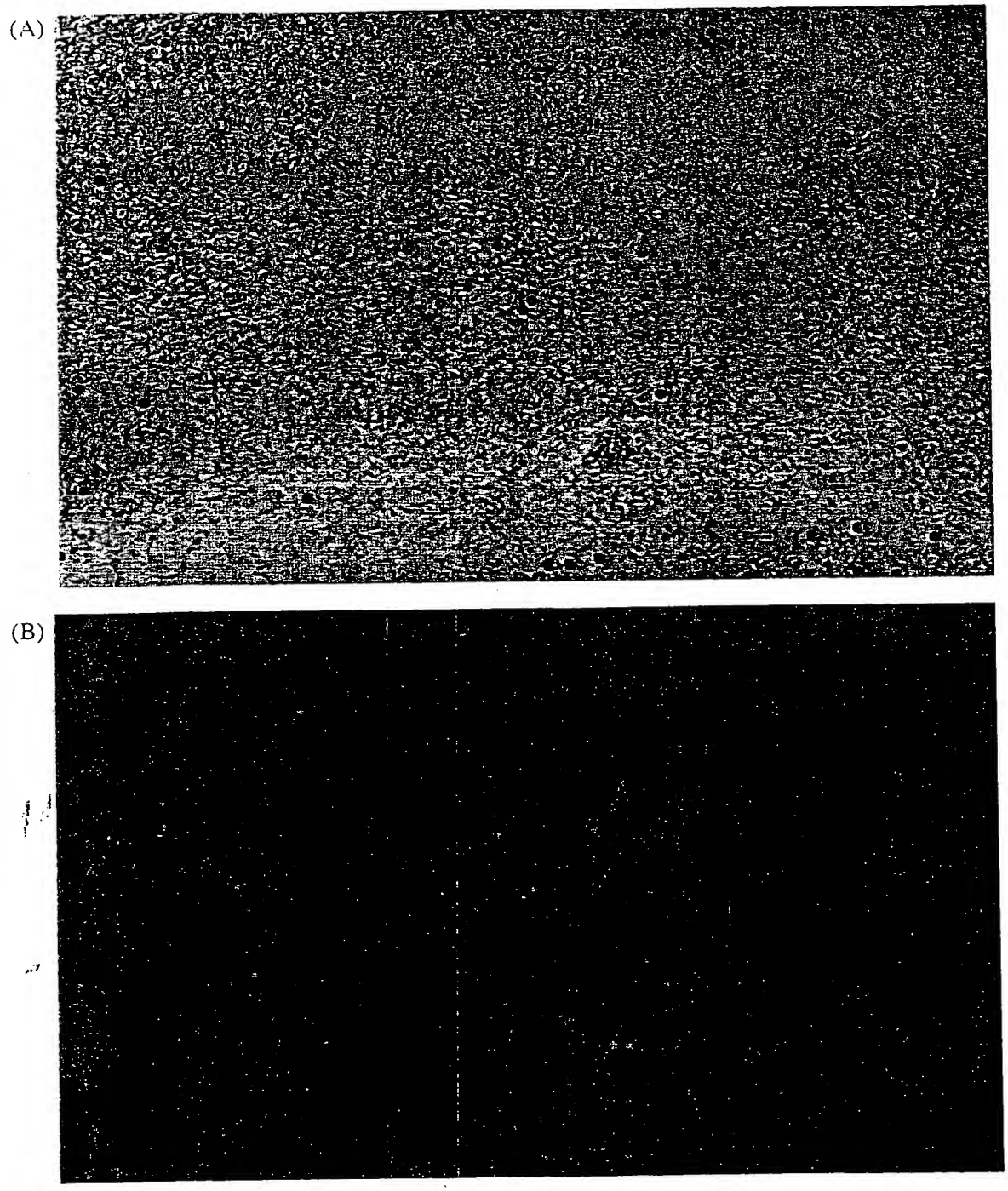

Fig. 4. Detection of DT40-hIgG3 cells in blood samples taken at the time of euthanasia of hens (17 days after initial injection of the cells). (A) Cells were observed by light microscopy and (B) by immunohistochemical staining for the presence of hIgG.

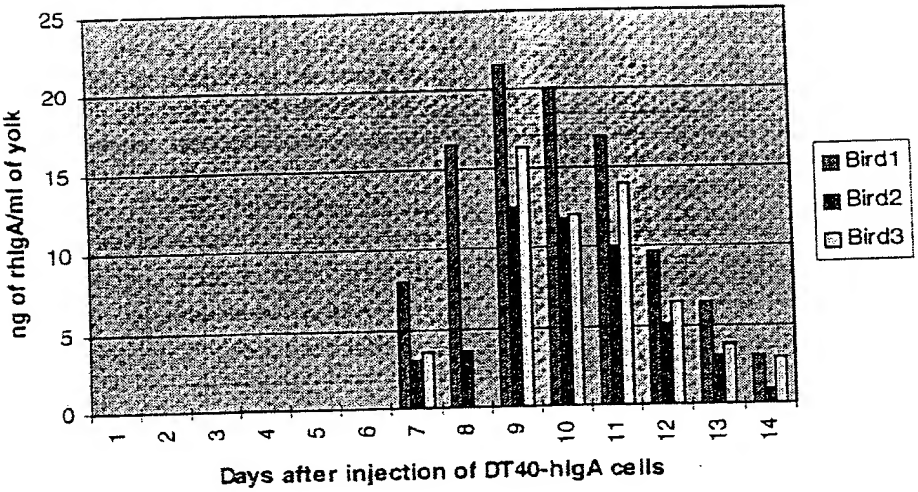

Fig. 5. Mean deposition of rhIgA per ml of yolk in eggs laid from hens ($n = 3$) intravenously injected with DT40 = hIgA cells, that did not develop tumors at the site of injection.

rhIgA would transport the rhIgA into the egg, 10^7 DT40-hIgA cells were intravenously injected into eight Hyline SC™ hens. Five of the hens injected with DT40 hIgA cells developed tumors at the site of injection. A low level of deposition of the rhIgA into the albumen was detected in all hens injected, but very little deposition of rhIgA was detected in the yolk of hens that developed tumors (data not shown). The three hens that did not develop any signs of tumor formation at the site of injection deposited up to 20 ng/ml rhIgA of yolk by Day 9 (Fig. 5).

4. Discussion

It has been recognized that the chicken oocyte plasma membrane contains IgY receptors (IgYR) that are saturable and specific, transporting maternal IgY into the developing follicle at the exclusion of maternal IgA (cIgA) and IgM (cIgM). Chicken IgM is similar to mammalian IgM, having four constant region domains and the characteristic COOH-terminal extension [14]. However, both the chicken γ and α constant regions contain four domains instead of the three domains found in their mammalian homologues [11,15]. For transgenic hens to be useful bioreactors in the production of rhAbs, avian B cells must be able to secrete functional rhAbs capable of being deposited into the egg despite the structural dissimilarities between chicken Ig and human Ig.

The deposition of intravenously injected hIgG into the egg yolk demonstrates that hIgG is capable of being sequestered into the developing avian follicle. If the blood volume of an adult hen is assumed to be 100 ml, hens injected with 10 μg of hIgG would initially have approximately 100 ng ml hIgG of serum. The deposition of 89 ng/ml of hIgG of yolk on Day 5 indicates that the yolk concentration of hIgG on this day is approximately equal to the serum concentration of hIgG on the day of injection. Assuming that hIgG is continually being both cleared metabolically and sequestered into all of the ovarian follicles, these data provide evidence that the oocyte receptor for IgY also recognizes rhIgG. Since the concentration of IgY in egg yolk of laying hens is approximately the same as the maternal serum concentration [1], it might also be inferred that hIgG is sequestered into the developing oocyte as efficiently as IgY. Therefore, although hIgG is structurally dissimilar to IgY, hIgG appears to possess the required domain(s) for binding the IgYR. The absence of hIgG in egg white is in agreement with previous work that suggested neither IgY nor hIgG is capable of binding the chicken secretory component [16] which appears to facilitate deposition of

cIgA and cIgM into the egg white. Intravenously injected hIgA was also deposited into egg yolk suggesting that unlike cIgA and cIgM, hIgA is capable of binding the IgYR. However, since hIgA is deposited with reduced efficiency compared to hIgG, it is probable that hIgA binds the IgYR with lower affinity than hIgG. The deposition of hIgA into egg white is consistent with evidence that hIgA, like cIgA and cIgM, can bind the chicken secretory component [16].

The arrangement and normal expression of a chicken Ig gene in transgenic mice [17] suggested that regulatory elements controlling Ig expression were conserved in avian and mammalian species. The ability of DT40-hIgG3 and DT40-hIgA cells to secrete functional rhAbs from a transgene under the control of a murine Ig promoter, further supports the suggestion that there is a conservation of regulatory elements involved in Ig gene expression in birds and mammals. We have concluded, therefore, that the avian B cell is an adequate environment for the production of rhAbs.

The deposition of rhIg into egg yolk upon the leukemia-like colonization of the hens by DT40-hIgG3 and DT40-hIgA cells, illustrates that transfected populations of avian B cells in hens can secrete functional rhIgG and rhIgA which are deposited into egg yolk. As in the case of injected hIgG, rhIgG3 produced in vivo by DT40-hIgG3 cells was deposited into egg yolk but not into egg white. Similarly, as in the case of injected hIgA, rhIgA produced in vivo by DT40-hIgA cells was deposited into both yolk and egg white. The deposition profiles of injected hIgA and hIgG and in vivo produced rhIgA and rhIgG suggest that both hIgA and hIgG contain homologues of the IgYR ligand and that the additional ability of hIgA to bind the chicken secretory component, results in the deposition of hIgA into the egg white. Therefore, it appears likely that the ability of human Ig of different isotypes to be deposited in either egg yolk or egg white is dependent on the molecule's ability to bind the IgYR or the chicken secretory component, respectively.

In vivo production of rhIgA and rhIgG elicits a chicken anti-human Ig response within 1 week of injection of the DT40 cells. It is not known whether chicken Ig/rhIg complexes are capable of binding the IgYR or if residual uncomplexed rhIg continued to persist for a few days after the detection of chicken anti-rhIg. Nevertheless, rhIg was detected in egg yolk for 3–4 days following detection of chicken anti-rhIg. The reason for the decline in rhIg deposition into egg yolk is unclear, but is likely partly explained by the developing chicken anti-human immune response.

Although the deposition of the rhAbs into the egg yolk in this model is modest, deposition of large amounts of rhAbs is not expected in view of the relatively low level of rhAb secretion by the transfected DT40 cells and the immune response against the rhAbs produced by the DT40 cells. In addition, although the hIgG seems to bind effectively to the IgYR, the concentration of rhAbs relative to IgY is very low and, therefore, the majority of the IgYR on the ovum will be bound to IgY rather than the rhAbs. Transgenic chickens would be expected to produce larger quantities of rhIg.

In conclusion, these studies demonstrate that avian B cells can produce human Ig in vivo and that the binding domain for the IgYR is possessed by both hIgA and hIgG enabling them to be sequestered by the developing ovarian follicle. Presumably, transgenic hens expressing rhAbs would not develop an immune response and deposition of the rhAbs would be maintained indefinitely. Further, by suppressing the expression of endogenous chicken Ig, it is conceivable that transgenic hens that deposit mg amounts of a single defined rhAb can be produced. This study indicates that the concept of harvesting therapeutically useful rhAbs from chicken eggs warrants further investigation and development. If the IgYR binding sites could be identified and characterized, it should be possible to direct the deposition of other therapeutically useful proteins into the egg yolk. Finally, these studies also suggest that DT40/SC Hyline chimeras may be novel models for studying in vivo trafficking of recombinant proteins produced from the stably transfected DT40 cells.

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